*Outline of*

# "Optimization of lipase production using oil cakes as substrate employing *Bacillus licheniformis* by Solid State Fermentation (SSF) Technique"

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# Optimization of lipase production using oil cakes as substrate employing *Bacillus licheniformis* by Solid State Fermentation (SSF) Technique.


## Abstract:

Lipases (E.C.3.1.1.3) are triacylglycerol acyl-hydrolase hydrolyze triacylglycerol into glycerol and fatty acids. They belong to the class of hydrolases. They are also responsible for the catalyzing the synthetic reactions like esterifications, interesterifications, and lysis of acids and alcohols. Presently lipases are widely used in industries like detergent, food modifications, chemical, and pharmaceuticals. Hence there is huge a market for lipases in the industries for bioprocessing, however, due to high market price or production cost limits the use of lipases. The aims of the present study are to produce a lipase on the cheaper media to lower the cost of production by optimizing techniques in solid-state fermentation techniques employing *Bacillus licheniformis* on various cakes of oil. Solid state fermentation is a method of fermentation of media in low free water activity or availability or fermentation of a moistened solids substrate. The solid substrate is acting as an adhesion or assistance matrix. Many reports show that Oil cakes are acting substrate medium for carbon and energy source and in some time in small amount as a nitrogen source. Solid state fermentation is majorly helpful for the developing countries or also in the developed countries in which biomasses are found in the lower cost. Additionally, it is found in many cases that in the solid-state cultivation the activity of lipase is higher than the other submerged fermentation or cultivations. Oil cakes are agro-industrial by-products produced in after the extraction of oil from seeds. Hence in the present work, the main focus will be on production of lipase by optimizing the process of solid state fermentation techniques by using *Bacillus licheniformis* optimization of factors affecting the lipase production and growth of *Bacillus licheniformis* on a fermentating oil cakes, optimization of oil cakes, optimization of lipase fermentation by mixed substrate solid state fermentation, estimation of nitrogen content.


## Introduction:

Applications of enzymes in the field of industrial processing have been significantly increased since the last few decades (Javed *et al*., 2018). The diverse applications of lipases force it for the production of the third highest used enzymes in the industrial field(Bharathi & Rajalakshmi, 2019). Lipases are enzymes of class hydrolases they hydrolyze the ester bonds of triacylglycerol and releases glycerol and fatty acids(Falony, Armas, Mendoza, & Hernández, 2006). Lipase catalyze the reactions like esterifications, hydrolysis,

interesterifications, alcoholysis, acidolysis, aminolysis (Javed *et al*., 2018) (Dutra & Terzi, 2008) It produced by plants, animals, microorganisms. Lipases are useful in the treatment of diseases in the humans like obesity (Mane & Tale, 2015). Interest in lipases are increasing for the biotechnology purpose due to their diverse and versatile properties day by day (Treichel, Oliveira, Mazutti, Luccio, & Oliveira, 2010).

Lipases are useful in pharmaceuticals for the production of anti-inflammatory drugs, in dairy for the formation flavored products, substitutes for the butter and additives for the cereals, snacks, and chewing, in detergent for the solubilizing grease and therefore removing of stains in tea processing, as an biosensors, oil biodegradation in production of biodiesel (Rigo *et al*., 2010) (Hasan, Shah, & Hameed, 2006). it is also used in the bakery industries for improving texture, in oil and fat industries for the production of polyunsaturated fatty acids, biodiesel from vegetable oils (Javed *et al*., 2018).

Besides all these uses the production cost of enzyme production is higher and hence need to be lower the cost for better use and this will be achieved producing lipases through the solid state fermentation than the submerged fermentation. Because of the higher activity, lower energy consumption, higher production, reduced wastewater output, easy to operate, and also achieved by agro-industrial waste a cheaper source (Liu *et al*., 2019).

Microorganisms commonly used for the production of lipases are fungi of the genus *Rhizopus, Aspergillus*, and *Penicillium*, yeasts *Yarrowia lipolytica*, *Candida rugosa*, and bacteria are *Bacillus, Burkholderia*, and *Pseudomonas* (Bharathi & Rajalakshmi, 2019) (Javed *et al*., 2018). The agro industrial waste like oil cakes of sunflower, cottonseed, sesame, mustard, groundnut, palm kernel, olive oil cake, are also majorly used in the production of lipases as they are rich source of protein and carbohydrates (Ramachandran, Kumar, Larroche, Ricardo, & Pandey, 2009). As they are wastes and found in the low cost they can be used for the production of mushroom, antibiotics, biopesticides, and other biochemical's using Solid state fermentation(Ramachandran *et al*., 2009) (Salihu *et al*., 2018). Oil cakes contains a oils which acting as a natural inducer for the production of lipases(Priji, Unni, Sajith, Binod, & Benjamin, n.d.).Scientists uses oil cakes as a industrial waste for the production of lipase enzyme (Selvakumar & Sivashanmugam, 2017).

Solid state fermentation is the fermentation process takes place at relatively low level of free water or water activity (Santis-navarro, Gea, Barrena, & Sánchez, 2011). Solid state fermentation process used for the production of mostly industrial products like enzymes (lipase, amylase, cellulose, inulinase, proteases, laccase, and many more), organic acids (citric acid, succinic acid, lactic acid), pigments like yellow, orange, biofertilizer and biopesticides (Thomas, Larroche, & Pandey, 2013). Although solid state fermentation is powerful tool for the production of industrial important products, there are some factors like temperature, pH, moisture content, aeration, C/N ratio of substrate, affects the production of products (Farinas, 2015).

The bacterial Lipases are produced by mainly bacillus species like *Bacillus subtilis, Bacillus licheniformis, Bacillus pumilis, Bacillus coagulans, Bacillus stearothermophillus*, some other species are also responsible for the production like pseudomonas sp.

Burkholderia sp and staphylococcal sp. (Bharathi & Rajalakshmi, 2019). Many reports shows that lipases from ***B****acillus licheniformis* shows significant lipase production (Javed *et al*., 2018) and it has higher thermo stability and alkaline tolerance produced from peanut oil cake (Annamalai, Elayaraja, Vijayalakshmi, & Balasubramanian, 2011). *Bacillus licheniformis* is a gram positive, chemoheterotrophic rod shaped bacteria "generally regarded as safe" (GRAS) (Inca-torres *et al*., 2018).

Hence the present work mainly focuses on the solid state fermentation for the production of lipases employing *Bacillus licheniformis* by using oil cakes as a substrate, which is considered as an agro waste. In the present study we are trying to work on solid state fermentation for the production of lipase through oil cakes by studying the factors in the oil cakes like substrate particle size, moisture content, nitrogen content, reducing sugar, C/N ratio, which affect the growth of microorganisms, study of environmental factors like $P^H$, Temperature, surfactants, inoculums size, effect of inducers for lipase production, optimization of lipase production by mixed substrate solid state fermentation, etc.

Hence from above, we are finally designed to target for the production, process optimization solid state fermentation, purification and characterization of microbial lipases employing *Bacillus licheniformis* by using an oil cake.

## Objectives:

Solid state fermentation has the immense importance in the biotechnical field for the production of different industrially potent products; hence in this study we majorly focused for the optimizing process parameters for the production of bacillus Licheniformis lipase. Hence the objectives designed for the study is as follows:

- Solid state fermentation of oil cakes (various substrates like sunflower, cottonseed, sesame, mustard, groundnut, palm kernel, olive oil cake will be tested) using *Bacillus licheniformis* for the lipase production
- Study of factors like substrate particle size, moisture content, nitrogen content, reducing sugar, C/N ratio affecting lipase production in the substrates.
- Optimization of lipase production in mixed substrate solid state fermentation.
- Study of factors like $P^H$, temperature, inoculums size, inducers for the lipase production, effect of surfactants.
- Optimization of oil cakes for lipase production.
- Estimation of oil cakes residues for nitrogen content as a protein source.
- Characterization of enzyme

## Methodology:

1) **Solid state fermentation of oil cakes (sunflower, cottonseed, sesame, mustard, groundnut, palm kernel, olive oil cake) using *Bacillus licheniformis* MTCC-10498 for the lipase production.**

   Bacterial culture of Bacillus licheniformis will be obtained from microbial type culture collection centre institute of Microbial Technology, Chandigarh, India. Activation of culture takes place through Luria bertaini (LB) broth for 24 hour (Inca-torres et al., 2018). After activation screening for the lipase activity checked by Tributyrin agar plates (Prasad & Sethi, 2013), after positive results solid state fermentation takes place by using given oil cakes, were done by using laboratory tray type bioreactors containing 15 g of solid oil cakes with 50 % (w/w) water added.

   Oil cakes were inoculated with Bacillus licheniformis MTCC-10498 a 10-7 spores/gram of dry solids (Oliveira et al., 2018). Lipase activity were determined by using standard condition with Tributyrin as a substrate in 30 ml of 2.5 mM Tris-HCl PH 8.2, 2mM CaCl2, 2mM NaDC or olive oil emulsion ( 10ml in 20 ml of 2.5 mM Tris–HCl pH 8.2, 2 mM CaCl2 , 4 mM NaDC) as substrate(fouzi, 2015).

2) **Optimization of factors like substrate particle size, moisture content, nitrogen content, reducing sugar, C/N ratio affecting lipase production in the substrates.**

   Optimization of factors like moisture content(Kunamneni, 2015), nitrogen content, reducing sugar, C/N ratio will be studied as per the Sharma C.K. (Sharma & Kanwar, 2012). Effect of incubation temperature and $P^H$ for production lipase will be varied from 35 to 60$^oC$ and $P^H$ varies from 5 to 10, (Annamalai *et al*., 2011).

   Moisture content will be determined by drying a measured amount of oil cakes and adding an water, after soaking calculation of moisture content by using formula

   (Percent of moisture content (initial) of solid medium =

   (Wt. of the wheat rawa - dry wt.) × 100/dry wt) (Kunamneni, 2015).

   The effect of nitrogen and sugar on production of lipase will be studied by adding sugar like glucose, sucrose, lactose, peptone, yeast extract, ammonium sulphate, at varying concentration from 1 -5 % (Bharathi, Rajalakshmi, & Komathi, 2018) (Rekha, 2012).

3) **Optimization of lipase production in mixed substrate solid state fermentation.**

   From the objective first the oil cakes which gives highest lipase activity will be mixed and in the different ratio (1:1, 1:2, 1:3) and estimated for the production of lipase (Ramachandran, Roopesh, & Nampoothiri, 2005).

4) **Study of factors like PH, temperature, inoculums size, inducers for the lipase production, effect of surfactants.**

   The study of external factors from the oil affects the growth of bacteria and also for the production of lipase enzyme. The vegetable oils like soybean, olive, corn, palm, castor are acting as an inducer for lipase production hence study finds the effect of oil on production of lipases (Dutra & Terzi, 2008). $P^H$ and temperature also affect the

growth and reproduction of bacteria and production of enzyme, the optimum conditions will be determined by incubating at different temperatures and $P^H$ (Annamalai *et al*., 2011). Varying concentration of volumes of active cultures will be used as inoculums from 0.2 to 1.6 % V/V these affect the production of lipase hence study trying to find out exact inoculums ratio for fermentation size (Ei-shafei & Rezkallah, 1997). Factors are studied by placket-burman design to screen which factor affect the production of enzyme (Salihu, Bala, & Alam, 2016) (Salihu *et al*., 2018).

5) **Optimization of oil cakes for lipase production.**
   On the studying all above factors optimizing the substrate and production of enzyme by taking cumulative optimized effect solid state fermentation of oil cakes will be done.
6) **Estimation of oil cakes residues for nitrogen content as a protein source.**
   Estimation of protein within fermented media for crude protein and fermented protein (Lowry's method) (Randall & Lewis, n.d.), total phosphorous compared to uninoculated feed (Ab, Vr, Sv, & Sd, 2019).
7) **Characterization of enzyme**
   The characterization of lipase will be done by studying the factors affect the enzyme activity like temperature, $P^H$, using lipolytic activity, substrate specificity, biocatalytic capacity in organic synthesis (Fouzi, 2015), determination of inhibitor and metal ions effect on lipase activity, determination of Triton X- 100 effects on lipase activity (Morais, Kamimura, & Ribeiro, 2016).

## Importance of the study:

Lipases are diverse in their enzymatic properties like they are acting in under delicate conditions, highly stable in organic solvents; possess substrate specificity and stereospecific or enantiospecific (Dutra & Terzi, 2008) (Hasan *et al*., 2006). Lipases are used in the fat and oil industry, dairy industries, bakery industries, leather processing, detergent industries, environmental bioremediation also in the pharmaceutical industries thus lipases are the third largest enzyme used in the industry after amylase and proteases (Aguieiras, Cavalcanti-oliveira, Cammarota, & Freire, 2018) (Neethu, Rahiman, Rosmine, Saramma, & Hatha, 2015) and also in the biotechnology field (Neethu *et al*., 2015). Due to the high production cost, there are limitations in the use of lipase. Many studies reveal that the substrate for the production of lipase has a great impact on increasing the price of enzyme and hence in the present study mainly focused on the production of lipases by using a cheaper substrate through solid state fermentation techniques.

Solid state fermentation is the main theme of the study is the optimizing processes of solid-state fermentation techniques which allow using better agro-industrial waste like oil cakes. Study indicates that the solid state cultivation is produced higher lipase activity than the submerged fermentation (Aguieiras *et al*., 2018). Oils in the medium also acting as an inducer in the lipase production and hence the present work focuses on the optimization of solid state fermentation technique for production of lipases from agro-industrial waste employing *Bacillus licheniformis*.

## Status of the problem:

The high enzyme cost, longer reaction time and lipase inhibition by the methanol and ethanol reduce the production and commercialization of biodiesel in the large scale (Aguieiras *et al*., 2018) the same problems in small variations occurred in the applications of lipase. Agro industrial wastes like oil cakes are found in cost effectives, but the process for the production of lipases thorough solid state fermentation (SSF) needs to be optimize. SSF is mainly attracts scientist for the production of value added products due to their cost effective methods of product recovery, ability to utilize discrete level of substrate for enzyme production. Drawbacks for the utilizing Solid state fermentation are few data available to optimize processes, growth and kinetic behavior of enzyme in SSF development by simple and practical methods (Wang & Yang, 2007).

## International status:

- Scientist now working in the immobilization of lipase enzyme for reuse the most stable enzymes.(Arana-peña *et al*., 2019)
- Immobilization of enzyme for the production of transestrification reactions for the production of biodiesel.(Guadix, Guadix, Guadix, & Emilia, 2019)

- *Bacillus licheniformis*–fermented products improve growth performance and the fecal microbiota community in broilers (Chen & Yu, 2019).
- Adejanildo da s Brazil studied on Mango agro-industrial wastes for lipase production from *Yarrowia lipolytica* and the potential of the fermented solid as a biocatalyst (Pereira, Ana, & Amaral, 2019).
- Multipurpose fixed-bed bioreactor to simplify lipase production by solid state fermentation and application in biocatalysis (Ávila, Gutarra, Fernandez-lafuente, Cavalcanti, & Freire, 2020).

### National status:

- Sequential optimization and large scale production of lipase using tri-substrate mixture from *Aspergillus niger* MTCC 872 by solid state fermentation(Mandari, Nema, & Devarai, 2019).
- Optimization and production of lipase enzyme from bacterial strains isolated from petrol spilled soil (Bharathi *et al*., 2018)
- Biodiesel production using lipase immobilized functionalized magnetic nanocatalyst from oleaginous fungal lipid (Jambulingam, Shalma, & Shankar, 2019).
- Biotransformation of rapeseed meal leading to production of polymers, biosurfactants, and fodder (Konkol, Szmigiel, Domżał-kędzia, Kułażyński, & Krasowska, 2019).

## Significance of the study:

Many research organizations are actively working in the field of solid state fermentations for lipase production thorough agro industrial waste and improvements in the increase in the lipase activity, now a day's many scientists' focus on the immobilization of enzyme using a nonomaterial. But these entire works endeavor for a single aims that are the cost-effectiveness of enzyme and reuse of the enzyme for processes. Also the present work is a contribution to the same goal, thorough optimizing the process of solid state fermentation. In the present study we are focusing on the process optimization for the production of lipases employing *Bacillus licheniformis* by solid state fermentation techniques.

## Work plan :

| Sr. No. | Objectives | Expected Time(Month) |
|---|---|---|
| 1. | Literature collection and review. | 0-6 |
| 2. | Solid state fermentation of oil cakes (sunflower, cottonseed, sesame, mustard, groundnut, palm kernel, olive oil cake) using *Bacillus licheniformis* for the lipase production | 6- 12 |
| 3. | Study of factors like substrate particle size, moisture content, nitrogen content, reducing sugar, C/N ratio affecting lipase production in the substrates. | 12-16 |
| 4. | Optimization of lipase production in mixed substrate solid state fermentation. | 16-20 |
| 5. | Study of factors like $P^H$, temperature, inoculums size, inducers for the lipase production, effect of surfactants. | 20-24 |
| 6. | Optimization of oil cakes for lipase production. | 24- 26 |
| 7. | Estimation of oil cakes residues for nitrogen content as a protein source.<br>Characterization of enzyme | 26-30 |
| 8. | Characterization of enzyme | 30-36 |
| 9. | Submission of thesis | 36-40 |

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|---|---|
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